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Molecular characterization of monopartite bhendi (*Abelmoschus moschatus*) yellow vein mosaic virus and screening of wild okra

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ABSTRACT

Bhendi yellow vein mosaic disease (BYVMD) is the most devastating viral disease of okra (*Abelmoschus moschatus* ssp. *moschatus*) which affects yield and quality of the produce in India. The causative agent of BYVMD is begomovirus i.e. bhendi yellow vein mosaic virus (BYVMV) having DNA-A molecule along with betasatellite. Present study was carried out during 2019–21 at research farm of ICAR-National Bureau of Plant Genetic Resources, New Delhi to find out the diversity of begomovirus and its satellite nucleotide sequences derived from wild okra infected samples exhibiting conspicuous symptom of BYVMD using PCR-based detection technique on two wild okra symptomatic samples, viz. EC361170 and EC361148. Full length amplification of BYVMV satellite molecule and partial amplification of DNA-A was carried out using PCR and cloning of both randomly selected samples showed the presence of monopartite BYVMV. In both samples, presence of DNA-A molecule, betasatellite and alphasatellite were noticed. Two year (*kharif* 2019 and 2021) field screening of 10 accessions of wild okra (*Abelmoschus moschatus* ssp. *moschatus*) was carried out at ICAR-NBPGR, New Delhi. Out of 10 accessions, viz. EC360586, EC360794, EC360830, EC360900, EC359730, EC359836, EC359870, EC360351, EC361111 and EC361171 screened, 4 accessions, viz. EC360794, EC360586, EC360830 and EC361171 showed resistant (R) reaction during both the seasons against BYVMD. This is the first study which showed the presence of alpha-satellite molecule of BYVMV from New Delhi region in wild okra along with its resistance source.

Keywords: Alphasatellite, Begomovirus, Betasatellite, Bhendi yellow vein mosaic disease, Wild okra

Okra [*Abelmoschus esculentus* (L.) Moench], popularly named as bhendi or lady's finger, belongs to the Malvaceae family and is cultivated throughout the world including India for vegetable purpose and has high nutritional value. Despite proper agronomical practices, okra yield is low due to several diseases, of which whitefly transmitted bhendi yellow vein mosaic (BYVM) and okra enation leaf curl disease (OELCuD) caused by distinctive mono and bipartite begomoviruses association with different DNA satellites (Alphasatellites and betasatellites) are major threat for its cultivation and production in India (Venkataravanappa *et al.* 2011). The BYVMV infected okra plants exhibit conspicuous symptoms including vein clearing, petiole bending, and chlorosis of infected leaves in advanced stage and ultimately cause reduction in plant growth and economic yield. It has been reported that an economic yield loss of 94–100% is observed if, plants are infected at 20 days old seedling stage (Pun and Doraiswamy 1999). Whereas, 49–84% yield loss was observed, if infection occurred at 50–65 days old plant after germination (Sastry and Singh 1974). There is a need to find out durable and sustainable resistant planting material to reduce future okra crop yield losses.

BYVMV belongs to the genus Begomovirus, family Geminiviridae (Fauquet and Stanley 2005). Genome structure is monopartite comprising of homologous DNA molecule and betasatellite (Jose and Usha 2003). The DNA-A molecule encodes seven open reading frames (V2–V1, C1–C5) in sense and antisense strands responsible for virus replication, transcription of gene and movement. Most of the old world monoparatite and few bipartite begomviruses are associated with sub-genomic molecule known as DNA satellites (alpha, beta and deltasatellites) (Briddon *et al.* 2001, Briddon *et al.* 2002, Venkataravanappa *et al.* 2011). Betasatellites are true ssDNA satellites, which depend on helper viruses for their replication, encapsidation, movement and vector transmission. Alphasatellites are not true satellites because they can replicate independently depend on the helper virus for movement, encapsidation and vector transmission. In the present study, molecular

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characterization of monopartite BYVMV and wild okra germplasm screening were carried out to find the durable source of resistance against BYVMD.

MATERIALS AND METHODS

Field screening of wild okra germplasm: Since New Delhi is a hot spot for bhendi yellow vein mosaic disease thus, field natural whiteflies predominance assisted disease transmission. A total of 10 accessions of wild okra, viz. EC360586, EC360794, EC360830, EC360900, EC359730, EC359836, EC359870, EC360351, EC361111 and EC361171 along with 4 susceptible checks (Arka Anamika, VRO 6, Pusa Sawani and Parbhani Kranti) were sown at research farm of ICAR-National Bureau of Plant Genetic Resources (NBPGR) (28°35'N and 77°12' E with an altitude of 228.6 m amsl), New Delhi, in randomized block design (RBD) during *kharif* 2019 and 2021 with 1 row for each accession with plant to plant spacing 30 cm × 30 cm and row to row spacing 75 cm × 75 cm.

Per cent disease incidence (PDI) was recorded on 10 plants of each accession. Observations were noted thrice at an interval of 25 days during vegetative crop growth stage. Yellow vein mosaic development on leaves was characteristic symptom of BYVMD. Disease scale (0–9) was followed (Mayee and Datar 1986) with slight modification on the basis of visual observations of okra plant.

$$\text{Per cent Disease Incidence (PDI)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants examined}} \times 100$$

Sample collection: Symptomatic and healthy okra leaves were collected from wild okra germplasm grown at research farm of ICAR-NBPGR. Two symptomatic wild okra accessions, viz. EC361170 and EC361148 were selected for collection of infected leaves exhibiting a typical bhendi yellow vein mosaic disease symptoms during *kharif* 2019 and 2021, whereas, EC360830 and EC361171 were used as a resistant (healthy) sample which was devoid of any symptom of bhendi yellow vein mosaic disease.

Okra genotypes field monitoring: Ten wild okra accessions as well as four okra checks (Arka Anamika, VRO 6, Pusa Sawani and Parbhani Kranti) were planted in *kharif* 2019 and 2021 to regularly monitor symptom variation and compare symptom progress in both wild as well as cultivated okra genotypes, from seedling stage till crop harvesting. Symptoms were monitored at regular intervals to understand host-virus interaction under field conditions and symptom development till the crop maturity.

Genomic DNA isolation: Total nucleic acid (DNA) was isolated from both symptomatic and healthy okra leaf samples using cetyl trimethyl ammonium bromide method (Doyle and Doyle 1990). Isolated genomic DNA was stored at -20°C for further polymerase chain reaction and cloning.

PCR and cloning of viral genome: Full length genome of BYVMV infecting wild okra and DNA satellite were amplified using primer pairs specifically targeted to DNA-A and DNA-B components (Venkataravanappa *et al.* 2012) and DNA satellite molecules (Briddon *et al.* 2002). The PCR amplification was carried out in thermal cycler (Bio-RAD DNA Engine). The total PCR reaction volume of 25 µl contained DNA template (100 pmol), Taq DNA polymerase (1.5 U), $MgCl_2$ (2.5 mM), dNTPs (2.5 mM), 25 pmol of each primer and nuclease-free water. PCR programme comprised of total amplification cycles of 35 with initial denaturation at 94°C for 3 min and final extension at 72ºC for 15 min. Amplification cycle conditions were denaturation at 94°C for 1 min, annealing at 55–59ºC (55ºC OY2395F/OY680R, 58ºC ToLCBD971F/ToLCBD2142R, 59ºC Beta01F/Beta02R and 56ºC alphaF/alphaR) for 45 sec, and extension at 72ºC for 90 sec. Amplified PCR products were electrophoresed (1 h at 80 volts) on 1% agarose gel and visualised in a gel documentation system. Full-length amplified products of DNA-A, beta and alphasatellite were cloned into the pTZ57R/T vector (Fermentas, Germany) as per manufacturer's instructions. The recombinant clones were confirmed by colony PCR using virus specific primers and restriction digestion. The three positive clones were sequenced at Agrigenome (ABA Biotech) sequencing facility, Kochi, Kerala, India.

Sequence analysis: Initially nucleotide sequences were checked using BLASTN search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF finder was used for ORF detection (https://www. ncbi.nlm.nih.gov/orffinder/). Protein encoding genes were translated using ExPASy proteomic server tool (https://web.expasy.org/translate/). Nucleotide sequences of betasatellite, alphasatellite and DNA-A molecules showed highest identity with present okra BYVMV isolates retrieved from the NCBI database. The pairwise per cent identity between okra BYVMV isolates and other selected Genbank isolates was calculated using sequence demarcation tool (SDT) (Muhire *et al.* 2014). Neighbor joining method was used to draw the phylogeny by employing MEGA-X (Kumar *et al.* 2018). A total of 21 alphasatellite reference sequences were used for developing dendrogram as well as SDT analysis (Table 1).

RESULTS AND DISCUSSION

Disease occurrence in germplasm: Field screening was conducted on the basis of appearance of disease symptoms and scoring done for BYVMD in wild okra accessions at New Delhi location during the main cropping seasons of *kharif* 2019 and 2021. Majority (>83%) of wild okra accessions exhibited typical bhendi yellow vein mosaic disease symptom during consecutive two years (*kharif* 2019 and 2021) of field screening.

During the first year of field screening (*kharif* 2019) average per cent disease incidence values were 12.08 with 0.274 CD (P=0.05) and 0.130 SEd. while during the second year (*kharif* 2021) average PDI values were 13.45 with 1.340 CD (P=0.05) and 0.633 SEd. which clearly indicated that disease progress was higher during the second year. Out of 10 accessions, four accessions, viz. EC360794, EC360586, EC360830 and EC361171 have PDI value of 4.753, 4.480, 5.180 and 7.333 respectively and showed resistant (R) reaction during *kharif* 2019 and *kharif* 2021 against bhendi

Table 1 Alphasatellites used in the sequence analysis with their respective abbreviations and GenBank accession numbers

Alphasatellite name	Accession number	Abbreviation
Bhendi yellow vein mosaic virus-associated alphasatellite isolate OK89B, complete sequence	KT390420.1	BYVMA
Bhendi yellow vein mosaic virus-associated alphasatellite isolate BYVMVA [Okra:Ropar:2010], complete sequence	JX183091.1	BYVMA
Bhendi yellow vein mosaic virus-associated alphasatellite, complete genome	NC018574.1	BYVMA
Sida yellow vein mosaic alphasatellite isolate OK101, complete sequence	KT390448.1	SiYVMA
Mesta yellow vein mosaic alphasatellite isolate OK113, complete sequence	KT390437.1	MeYVMA
Mesta yellow vein mosaic alphasatellite isolate OK109, complete sequence	KT390434.1	MeYVMA
Mesta yellow vein mosaic alphasatellite isolate OK104, complete sequence	KT390431.1	MeYVMA
Mesta yellow vein mosaic alphasatellite isolate OK102, complete sequence	KT390430.1	MeYVMA
Mesta yellow vein mosaic alphasatellite isolate OK97, complete sequence	KT390429.1	MeYVMA
Mesta yellow vein mosaic alphasatellite isolate OK96, complete sequence	KT390428.1	MeYVMA
Croton yellow vein mosaic alphasatellite isolate OK91B, complete sequence	KT390424.1	CYVMA
Okra leaf curl alphasatellite isolate OK92, complete sequence	KT390425.1	OLCuA
Okra leaf curl alphasatellite isolate OK90A, complete sequence	KT390421.1	OLCuA
Okra leaf curl alphasatellite isolate OK86, complete sequence	KT390415.1	OLCuA
Okra leaf curl alphasatellite isolate OY62, complete sequence	KT390414.1	OLCuA
Guar leaf curl alphasatellite isolate OK95, complete sequence	KT390427.1	GLCuA
Guar leaf curl alphasatellite isolate OK91A, complete sequence	KT390423.1	GLCuA
Guar leaf curl alphasatellite isolate OK111, complete sequence	KT390435.1	GLCuA
Guar leaf curl alphasatellite isolate OK108, complete sequence	KT390433.1	GLCuA
Tobacco curly shoot alphasatellite isolate OK112, complete sequence	KT390436.1	TbCSA
Ageratum enation alphasatellite isolate OK106, complete sequence	KT390432.1	AEA

yellow vein mosaic disease.

Symptom variation under natural/field conditions: Ten wild okra germplasm lines were monitored for symptom variation and host-virus interaction along with four cultivated okra lines. First type, very early infection of the leaves of young plants in the season resulted in complete yellowing of leaves which later turned brown and dry at the end for both cultivated as well as wild okra germplasm. Second type, if infection develops after flowering, then floral parts and upper leaves conspicuously exhibit vein clearing symptoms and infected plant produce few okra capsules which turn yellow and hard at picking stage.

There are different factors involved in field for symptoms variation which includes type of virus strain, infection time, biotypes of whiteflies vector, plant genotype and varying environmental conditions (Polston and Anderson 1997).

Detection of bhendi yellow vein mosaic virus (BYVMV): Sequences of begomovirus DNA-A and DNA-B components from field samples were amplified using a primer pair OY2395F/OY680R and ToLCBD971F/ToLCBD2142R, respectively. Amplification was observed for OY2395F/OY680R primer pair while amplification was absent using ToLCBD971F/ToLCBD2142R set of primer.

PCR amplification of BYVMV DNA-A molecule using OY2395F/OY680R degenerate primer pair amplified at 1.2 kb for two randomly collected symptomatic accessions, viz. EC361170 and EC361148 of wild okra whereas amplification was absent for two randomly selected resistant (healthy) wild okra accessions, EC360830 and EC361171 .

PCR product of 1.2 kb was sequenced and found 98.16% sequence similarity with BYVMV, which confirmed that BYVMV is a monopartite begomovirus having association of satellite molecule. Sequence generated in this study for partial DNA-A region of virus isolate is submitted in Genbank database under the accession number OK067306 (Table 2).

Table 2 Sequences generated in this study with their respective abbreviations and GenBank accession numbers

Begomovirus sequences	Accession number	Abbreviation
Bhendi yellow vein mosaic virus New Delhi isolate, host- wild okra [*Abelmoschus moschatus* ssp. *moschatus*], partial DNA-A sequence	OK067306	BYVMV
Bhendi yellow vein mosaic virus New Delhi isolate, host- wild okra [*Abelmoschus moschatus* ssp. *moschatus*], partial betasatellite sequence	OK067305	BYVMB
Okra leaf curl virus alphasatellite New Delhi isolate, host-wild okra [*Abelmoschus moschatus* ssp. *moschatus*], complete alphasatellite sequence	MW699036	OLCuA

PCR amplification, cloning and sequence analysis of satellite molecule: Sequence of satellite molecule (beta and alpha) from infected field samples were amplified using a primer pair Beta01F/Beta02R and alphaF/alphaR for betasatellite and alphasatellite, respectively. Amplicon of 1.3 kb was obtained for both the molecules from two symptomatic accessions, viz. EC361170 and EC361148 of wild okra while in healthy leaf samples of accessions, viz. EC360830 and EC361171 the amplicon was absent, indicating accessions were free from the BYVMV.

The PCR amplified products of betasatellite and alphasatellite were cloned into pTZ57R/T vector and sequenced. Presence of betasatellite (1.3 kb) viral molecule showed similarity (97.81%) to BYVMV isolate while alphasatellite (1.3 kb) viral molecule showed 95.71% sequence similarity to okra leaf curl virus (OLCuV) isolate in BLAST analysis. Sequence generated for betasatellite and alphasatellite molecule in this study was deposited in Genbank database under the accession number OK067305 and MW699036, respectively (Table 2).

The comparison of genome sequences of selected alphasatellite molecule of begomovirus revealed that it shared highest sequence similarity with okra leaf curl alphasatellite molecule. There are three major clusters obtained in the dendrogram and the sequences generated in this study were found closest with okra leaf curl alphasatellite (OLCuA) having accession number of KT390414.1 infecting okra. Phylogenetic dendrogram showing the relationship of okra leaf curl alphasatellite (OLCuA, this study) complete genomes with other isolates of BYVMA, SiYVMA, MeYVMA, CYVMA, OLCuA, GLCuA, TbCSA and AEA. Star symbol indicate sequences obtained from samples exhibiting bhendi yellow vein mosaic disease (BYVMD) (Fig 1).

Two complete sequences of begomovirus alphasatellite were amplified, cloned and one representing sequence was submitted in Genbank. Sequence generated in this study i.e. OLCuA (MW699036) shared maximum identity 90% with OLCuA (KT390414.1) in Sequence Demarcation Tool based pairwise sequence comparisons. Colour-coded pairwise identity matrix was generated from 21 begomovirus alphasatellite complete genomes (bhendi yellow vein mosaic alphasatellite, sida yellow vein mosaic alphasatellite, mesta yellow vein mosaic alphasatellite, croton yellow vein mosaic alphasatellite, okra leaf curl alphasatellite, guar leaf curl alphasatellite, tobacco curly shoot alphasatellite and ageratum enation alphasatellite). Each coloured cell represented a share identity score between 2 sequences (one

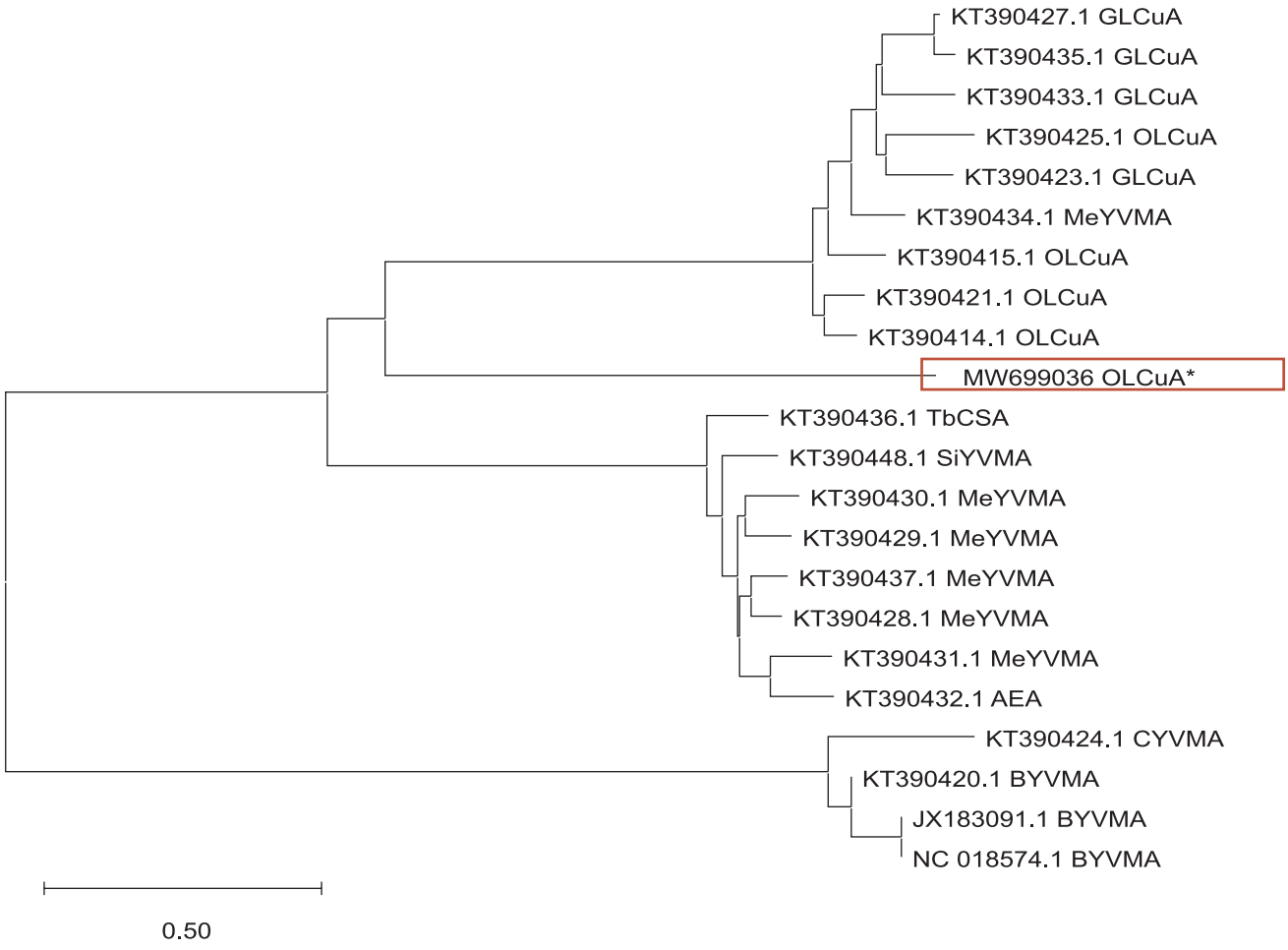

Fig 1 Phylogenetic dendrogram showing the relationship of Okra leaf curl alphasatellite (OLCuA, this study) complete genomes with other isolates.

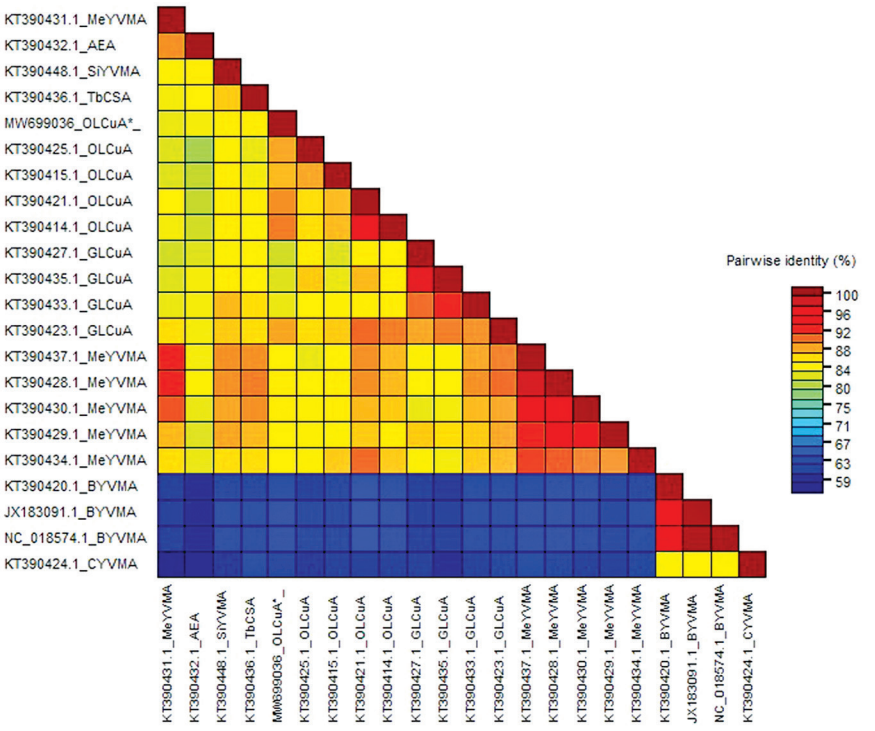

Fig 2 Sequence Demarcation Tool based pairwise sequence comparisons. Colour-coded pairwise identity matrix generated from 21 begomovirus alphasatellite complete genomes.

indicated horizontally to the left and also the alternative vertically at the bottom). A coloured key indicates the correspondence between pairwise identities and also the colours displayed within the matrix. The sequences generated by this study are indicated by star symbol (colour figure online) (Fig 2).

Data availability: Partial DNA-A full length nucleotide sequences generated in this study are given accession number OK067306 from NCBI whereas nucleotide sequences of betasatellite are provided accession number OK067305 while full length nucleotide sequences of alphasatellite were submitted to GenBank and given accession number MW699036.

Bhendi yellow vein mosaic disease is considered as the most widely spreading disease throughout India due to newly emerging begomovirus which resulted in reduced yield as well as poor quality okra capsule (Capoor and Varma 1950, Singh 1980). Generally, betasatellite associated monopartite begomovirus species are responsible to develop BYVMD in okra crop.

Globally, different species of begomoviruses infecting okra have been well documented in different parts of the world (Venkataravanappa *et al.* 2012). Besides these, bhendi yellow vein Delhi virus, a new bipartite begomovirus species and bhendi yellow vein mosaic virus (BYVMV) having DNA-B of tomato leaf curl New Delhi virus (ToLCNDV) have been well documented (Venkataravanappa *et al.* 2012). Yellow vein mosaic disease of okra caused by BYVMV was first reported from Bombay Presidency (Kulkarni 1924). Subsequently, it has been reported that BYVMD is caused by several begomoviruses in India (Usha 1980). Most of the begomoviruses that have been reported so far from malvaceous crops are monopartite and are associated with a betasatellite and alphasatellite molecule (Saunders *et al.* 2000, Briddon and Stanley 2006, Venkataravanappa *et al.* 2011).

In many parts of the world BYVMD is reported to be caused by complex of different begomovirus species (Briddon *et al.* 2001, Bull *et al.* 2006, Zhou *et al.* 2008). These begomovirus complexes take part in inter-species recombination which seem to play a vital role in begomovirus evolution in okra. It is quite possible that, inter-species recombination in the past (50 years) would have directly been involved in emergence of multiple new begomoviruses and associated diseases have been well documented in many crops (Berrie *et al.* 2001, Schnippenkoetter *et al.* 2001).

In the present study, presence of monopartite BYVMV having association of betasatellite and alphasatellite from wild okra was also noticed. A rich diversity of viruses infecting okra is of major concern, therefore, it is utmost needed to find out a durable resistance source against BYVMD. In our field experiment, we have observed four lines, viz. EC360794, EC360586, EC360830 and EC361171 resistant to BYVMV which can be further utilized in resistance breeding programmes.

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